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2 ***Paratrichodorus divergens* sp. n., a new potential virus vector of**
3 **tobacco rattle virus and additional observations on *P. hispanus* Roca &**
4 **Arias, 1986 from Portugal (Nematoda: Trichodoridae)**

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Summary - During a survey of trichodorids in Continental Portugal, a new trichodorid species, *Paratrichodorus divergens* sp. n., was found. It is described and illustrated with specimens from the type locality and additional morphometric data and photographs of specimens obtained from soil samples collected in seven other localities are also included. The species is characterised in female by distinct drop-like to triangular oblique vaginal sclerotisations diverging outwards, sperm cells usually distributed all through the uteri, and in male by thin, almost straight, striated spicules, the two posteriormost pre-cloacal supplements relatively close to one another and usually opposite the distal third of retracted spicules, a slightly bilobed cloacal lip and sperm cells with sausage-shaped nucleus. *Paratrichodorus divergens* sp. n. most closely resembles *P. hispanus* Roca & Arias, 1986 with which it often occurs in mixed populations. Additional information is also provided for *P. hispanus*. For the new species, coding of the features are added following Decraemer and Baujard's identification key.

Keywords – description, taxonomy, morphology, nematode, trichodorid, new species.

During a survey of trichodorid nematodes from different vegetation types and habitats conducted in Continental Portugal in 1982-1988, various populations of a new *Paratrichodorus* species were found. Up to now, the species was referred to as *Paratrichodorus* sp. C (Almeida, 1993; Duarte *et al.*, 2001). The presence of mixed trichodorid populations made identification more difficult, especially as specimens were often present in small numbers. *Paratrichodorus divergens* sp. n. is the most widespread *Paratrichodorus* species in Portugal (Almeida, 1993) and occurs very frequently in mixed populations with *P. hispanus* Roca & Arias, 1986. Attempts were previously made to differentiate these two species (Almeida *et al.*, 1998). The new species was detected in 1987 by the senior author and since then it has been collected every year from the type locality. *Paratrichodorus divergens* sp. n. is described from the type population on Granjas farm, near Braga, Minho Region (North Portugal). Additional data are also provided for populations of this new species from seven other localities and also for four populations of *P. hispanus* from some of these sites.

Currently, six species belonging to the genus *Paratrichodorus* are known from Continental Portugal: *P. allius* (Jensen, 1963) Siddiqi, 1974, *P. anemones* (Loof, 1965) Siddiqi, 1974, *P. hispanus*, *P. minor* (Colbran, 1956) Siddiqi, 1974, *P. nanus* (Allen, 1957), Siddiqi, 1974 and *P. pachydermus* (Seinhorst, 1954) Siddiqi, 1974.

An accurate identification of species of the genus *Paratrichodorus* is of most importance as some are virus vectors. In particular *P. hispanus* is already known to transmit *Tobacco rattle virus* (TRV), in Portugal (Ploeg & Brown, 1997; Taylor & Brown, 1997; Brown & Weischer, 1998) and studies on the possible transmission of TRV by *Paratrichodorus divergens* sp. n. by molecular characterisation of isolates and associated vectors are in progress (Duarte *et al.*, 2002; Duarte, pers. comm.).

Materials and methods

The nematodes were extracted from soil by a modification of the decanting and sieving method of Alpey and Boag (1976), killed by gentle heat in water, fixed in triethanolamine formalin water solution (TAF), processed to anhydrous glycerin by the

glycerol-ethanol method and mounted on Cobb slides, using the wax-ring method with paraffin (M.P. 56-58 °C) (Cobb, 1917; Courtney *et al.*, 1955; Seinhorst, 1959; De Maeseneer & D' Herde, 1963; Hooper, 1986).

Light microscopy (LM) studies, drawings and photographs, were made using a Leitz Dialux-20 microscope provided with a camera lucida and a differential interference contrast (DIC) equipment (Nomarski, 1955).

Coding of the features of *Paratrichodorus divergens* sp. n. according to the polytomous identification developed by Decraemer and Baujard (1998) is provided.

Scanning electron microscopy (SEM) studies were conducted on specimens relaxed and killed by gentle heating in a drop of tap water, transferred to TAF and then to cold 2% glutaraldehyde prepared with 0.1M sodium cacodylate buffer at pH 7. Fixed nematodes were washed in this buffer and postfixed in 2% osmium tetroxide (OsO₄), rinsed again in the buffer solution and dehydrated, at 15 min intervals, through 5, 10, 25, 50, 75, 95 and 100% (three times) ethanol. They were then critical point dried in liquid carbon dioxide (CO₂), mounted on SEM stubs standing in a piece of hair, sputter-coated with gold and scanned using a JEOL JSM – 630 1F at 15 kV accelerating voltage. Photomicrographs were improved using an airbrush technique (Eisenback, 1985).

Soil texture was based on the conventional soil textural classes triangle adapted by Pereira Gomes and Antunes da Silva (Costa, 1999).

***Paratrichodorus divergens** sp. n.**

= *Paratrichodorus* sp. C *apud* Almeida (1993), Duarte *et al.* (2001)

(Figs 1-4, 8, 9)

MEASUREMENTS

See Table 1 for females and Table 2 for males.

*The species name refers to the quite characteristic morphology of the vulval sclerotised pieces of females in lateral view, which appear with tips diverging away from vulva.

DESCRIPTION

Female

Females usually in about equal or slightly higher numbers than males. General appearance typical for the genus. Body medium-sized, straight or slightly curved ventrally. Lip region with distinct protruding double papillae formed by two subdorsal and two subventral papillae of second, and four submedian cephalic papillae of the third, circlet. Amphidial aperture sublabial, elliptical. Amphidial sensillae pouch conspicuous, typical for the genus. Body cuticle clearly swollen after fixation, 3.5-7 μm thick at mid body with two different optical layers, a thicker outer one (2.5-5 μm) and a thinner inner one (0.8-2 μm); base of inner cuticular layer slightly transversely striated. Stoma tubular, with distinct sclerotised rods, 5 (4-6) μm long. Onchiostyle ventrally curved, medium-sized. Guide ring around distal third of onchiostyle, at 23 (20-25) μm from anterior end. Pharynx gradually widening posteriorly to a bulb with five gland nuclei; bulb occupying less than two third of neck length. Position of dorsal gland nucleus variable, mostly located at posterior half of bulb, *i.e.*, at level of, or just anterior to, posterior pair of large ventro-sublateral gland nuclei; small anterior ventro-sublateral gland nuclei often difficult to observe. Dorsal overlap of pharyngeal bulb by intestine 16 (8-26) μm long. Nerve ring at level of anterior part of isthmus, at 67 (61-77) μm from anterior end. Secretory-excretory (S-E) pore situated at level of posterior isthmus region. Reproductive system didelphic, amphidelphic, with reflexed ovaries, without spermathecae; sperm, when present, dispersed throughout short uteri. Sperm cells large, with sausage-shaped nucleus. Vulva at mid-body, pore-like (0.2-0.4 μm diameter) or sometimes observed as a minute (1 μm) transverse slit in ventral view. Vagina 6-9 μm long, extending inwards over 27.9-51.6% of the corresponding body diameter. Vaginal sclerotisations in lateral optical section quite evident, appearing as large drop-like or oval-triangular oblique pieces (2-3 X 1-1.5 μm), with tips diverging outwards, *i.e.*, away from the vulva and 4 to 5.5 μm apart. Refractive structure present at the distal end of

vagina (Fig. 1 C) in most females with sperm (five out of eight females). Tail rounded, with one pair of subterminal pores. Anus subterminal.

Male

General morphology similar to female for most characters. Body straight or slightly curved ventrally. One distinct ventromedian cervical papilla (CP) present posterior to onchiostyle region and anterior to secretory-excretory pore. Secretory-excretory pore located anterior two-thirds of pharyngeal bulb. Nerve ring at 57-74 μm from anterior end. One pair of lateral cervical pores present in variable positions: mostly slightly anterior to CP ($n = 20$); *ca* 20 μm anterior to CP ($n = 4$); opposite to CP ($n = 3$); between CP and S-E pore ($n = 2$); posterior to S-E pore ($n = 1$). Dorsal intestinal overlap over posterior pharyngeal bulb 8-25 μm long. Testis single, outstretched. Sperm cells large, with long sausage-shaped nucleus (7-10 X 2-4 μm). Three precloacal ventromedian supplements (SP): anteriormost (SP3) weakly developed; posteriormost (SP1) and median one (SP2) opposite posterior third of retracted spicules, with SP2 up to mid-region of retracted spicules in some specimens. Distance between SP1 and SP2 1-1.5 times distance between SP1 and cloacal opening (CL); anteriormost supplement at 96.3 (83-128) μm from cloacal opening, *i.e.*, usually about twice distance between cloacal aperture and head of retracted spicules. One male with four precloacal supplements: CL-SP1 = 8 μm ; SP1-SP2 = 18 μm ; SP2-SP3 = 38 μm ; SP3-SP4 = 53 μm . Spicules nearly straight, manubrium slightly widened and offset; shaft irregularly outlined in anterior part, quite narrow (1.4–1.8 μm greatest width at mid-spicule) and finely striated. Gubernaculum with distal half thickened. Tail broadly rounded. One pair of well developed postcloacal subventral papillae and one pair of subterminal pores present. Caudal alae weakly developed. Anterior cloacal lip bilobed flap-like, protruding for about 1 μm over cloacal opening. Some subtle superficial cuticular irregularities subventrally anterior to cloacal opening (SEM; Fig. 9 B).

TYPE HABITAT AND LOCALITY

A sandy loam acid soil (pH about 5) around the roots of arum lily, *Zantedeschia aethiopica* Spreng and ivy, *Hedera canariensis* Willd, next to a stone wall close to an old fallow potato field at Granjas Farm, Esporões, Braga, in Minho Region, North Portugal, at about 200 m altitude.

TYPE MATERIAL

Holotype female on slide UM.DB-T 200 deposited in the collection of the Departamento de Biologia, Universidade do Minho, Braga, Portugal. Paratypes: one male deposited in collection of Departamento de Biologia, Universidade do Minho, Braga, Portugal; two males and two females at collection of Nematology Laboratory of Departamento de Zoologia, Universidade de Coimbra, Portugal; two males (slide RIT 696) and two females (slides RIT 697, 698) in the nematode collection of the Royal Belgian Institute of Natural Sciences, Brussels, Belgium; two males and two females in collection of the Scottish Crop Research Institute, Dundee, Scotland, UK. All other type specimens deposited (females and males) in the collection of the Departamento de Biologia, Universidade do Minho, Braga, Portugal.

OTHER HABITATS AND LOCALITIES

From sandy loam soil (pH 4.5) of a fallow potato field at Granjas Farm, Esporões, Braga, at 200 m altitude, in 1987; from sandy loam soil (pH 4.1) in association with rye-grass, *Lolium multiflorum* L. and rye, *Secale cereale* Lam., in Espinheiro, Celorico da Beira (Centre of Portugal), at 410 m altitude, collected in 1982 and 1986; from soil (pH 5.2) with hop, *Humulus lupulus* L., in Areias de Vilar de Frades, Barcelos (North), at 100 m altitude, in 1987; from loam soil (pH 5.4) with bean, *Phaseolus vulgaris* L., in Gafanha da Boavista, Aveiro (Centre), at 5 m altitude, in 1985; from silt loam soil (pH 4.2) with vine *Vitis vinifera* L., in Escudeiros, Braga (North), at 200 m altitude in 1987; from loam soil (pH 4.3) with pine, *Pinus pinaster* Ait., Barco, Barcelos (North), at 200

1 m altitude, in 1987; from loam soil (pH 4) of a fallow potato field, in Forjães,
2 Esposende (North), at 100 m altitude, in 1987; from silt loam soil (pH 4.4) with vine, *V.*
3 *vinifera* L., in Paço de Calheiros, Calheiros, Ponte de Lima (North), at 175 m altitude, in
4 1987. All samples from Continental Portugal were collected by the senior author.

5 More recently the new species was recorded from still other localities in samples
6 collected in potato fields where symptoms of TRV in the tubers were detected (Duarte
7 *et al.*, 2001): Lugar do Bustelo, Duas Igrejas, Vila Verde, Braga (North), in March 1999
8 and June 2000; Vila Verde, Oliveira do Bairro (Centre), in March 1999; Carromeu,
9 Mira (Centre), in June 2001; Quintal da Capela, Alijó, Vila Real (North), in June 1999
10 and November 2000.

11 12 13 DIAGNOSIS AND RELATIONSHIPS 14

15 *Paratrichodorus divergens* sp. n. is characterised by the following characteristics:
16 females with well developed, triangular/drop-like oblique vaginal sclerotised pieces in
17 lateral view, with tips diverging away from the vulva and 4.5-5 μm apart; sperm
18 dispersed throughout the uteri; males with quite thin and nearly straight, 47-55 μm long
19 striated spicules, one ventromedian cervical papilla, a pair of lateral cervical pores and
20 three precloacal supplements, of which the two most posterior are quite close to one
21 another, both lying opposite the posterior third of the retracted spicules, sperm cells
22 large with sausage-shaped nucleus and slender caudal alae.

23 *Paratrichodorus divergens* sp. n. closely resembles *P. hispanus* and in the past
24 has often been identified as the latter, moreover since both species regularly occur
25 together in mixed populations. However *P. divergens* sp. n. differs from *P. hispanus* in
26 that the females have the vulval sclerotised pieces with tips diverging away from the
27 vulva *vs* tips pointing towards the vulva in *P. hispanus* and by sperm cells dispersed
28 along the uteri *vs* packed in the spermathecae in *P. hispanus* and in males by possessing
29 quite thin (greatest width at middle 1.8 μm) spicules *vs* stouter (greatest width at middle
30 4 μm) and longer spicules than *P. hispanus*, a shorter onchiostyle than in *P. hispanus*
31 (Table 6), sausage-shaped sperm nucleus longer than *P. hispanus*, distance between
32 cloacal opening and posteriormost precloacal supplement short and similar to the

distance SP1-SP2 vs usually not similar distances and longer distance SP1-SP2 in *P. hispanus*, caudal alae less developed (expanding since mid-region of head of retracted spicules) than in *P. hispanus* (starting its expansion anteriorly to this region) and cloacal lip bilobed and protruding for about 1 μm over the cloacal opening vs for about 1-3 μm in *P. hispanus*.

Additionally *Paratrichodorus divergens* sp. n. is compared with *P. hispanus* (published data) and related species: *P. anemones*, *P. pachydermus* and *P. weischeri* (Table 3). For practical purposes, species are not listed in alphabetical order.

CODIFICATION IN THE POLYTOMOUS KEY

A new code for feature M, *i.e.*, orientation of vaginal sclerotised pieces, has been introduced: code 5 = oblique with tips divergent away from the vulva. In addition to the divergent orientation, the sclerotised pieces can be considered as widely apart.

The code in the polytomous key for identification of species of the family Trichodoridae (Decraemer & Baujard, 1998) for females is: A 213, B 20, C 1, D 1, E 200, F 300, G 1, H 26, I 12, J 11, K 300, L 3, M 5, N 1, O 22, P 11, Q 4, R 22 and S 1; for males is: A 210, B 22, C 22, D 1, E 0, F 3 (4), G 22, H 22, I 33, J 100, K 22, L 77, M 270, N 11, O 100, P 1.

Using the polytomous key (Decraemer & Baujard, 1998) for *Paratrichodorus* males, and considering prime characters D (number of CP), E (number of CP within the onchiostyle region), and L (shape of spicule shaft), all of which are easy to observe and usually constant within species, groups *P. divergens* sp. n. together with *P. hispanus* and *P. weischeri* Sturhan, 1985. *Paratrichodorus weischeri* differs from the new species mainly by shorter spicules 34-37 vs 45-56 μm and different distribution of SP2, *i.e.*, near the spicule head vs SP2 close to SP1 and within the posterior half of retracted spicules. For females, the use of the orientation of sclerotised pieces (prime character M) will differentiate the new species from all other species of the genus.

1
2 ***Paratrichodorus hispanus* Roca & Arias, 1986**

3 (Figs 5-9)

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6 **MEASUREMENTS**

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8 See Table 4 for females and Table 5 for males.

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11 **Remarks**

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13 Morphological data on the Portuguese populations are consistent with those
14 described for *P. hispanus* type population (Roca & Arias, 1986). All populations had
15 sperm grouped in spermathecae in females and stout spicules with 2-4.5 μm greatest
16 width at mid-spicule and obvious flap-like bilobed anterior cloacal lip in males. Strong
17 superficial cuticular irregularities were observed ventrally and subventrally on the
18 posterior region of male by SEM (Fig. 9 C, D, arrows).

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21 **Discussion**

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23 *Paratrichodorus divergens* sp. n. and *P. hispanus* have been most frequently
24 found to occur in Continental Portugal as mixed species populations, usually with few
25 individuals (Almeida, 1993; Almeida *et al.*, 1998; Duarte *et al.*, 2001; Queirós, 1997).
26 Distinguishing between the two species is easiest when females are present and are
27 observed in lateral view. In general, morphological characters are more obvious than
28 morphometric data, where some overlapping occurs for the various features. Additional
29 morphometric and morphological data to the type population were provided for a
30 Spanish population of *P. hispanus* found in the Province of Jaén, Sierra Nevada, in
31 Southern Spain (Decraemer *et al.*, 1993).

32 The following characters and morphometrics in *P. divergens* sp. n. separate this

new species from the *Paratrichodorus* Spanish population of Jaén and the type population of *P. hispanus* (Table 6). Body length of *P. divergens* sp. n. is mostly shorter and closer to specimens from Jaén than to type specimens of *P. hispanus*; the onchiostyle length in females and males of *P. divergens* sp. n. is also more similar to that of the Jaén population (slightly shorter than in the type population of *P. hispanus*); the secretory-excretory pore position in specimens from the new species is more anterior and similar to data for specimens from Jaén than that from *P. hispanus* type population; male spicule length in the new species is more similar to that in Jaén population and shorter compared with the type population of *P. hispanus*; the short distance between SP1 and SP2 in the new species, similar to that between cloacal opening and SP1, resembles that of the Jaén males (in the type population of *P. hispanus* distance between SP1 and SP2 is sometimes double or greater than the distance Cl-SP1).

Based on morphometric data, the Jaén population occupies an intermediate position between the new species and *P. hispanus*, though closer to the former. Unfortunately, the study on the Jaén population did not include illustrations of specimens (Decraemer *et al.*, 1993). The photographic illustrations of *P. hispanus* female in Decraemer (1995) are from *P. hispanus* type specimens (Decraemer, pers. comm.). It must be emphasised some differences referred above between the Jaén and the type population of *P. hispanus* were noted by Decraemer (1995) who very accurately presented them as additional remarks to this species. Those data may suggest that the Jaén study of Decraemer *et al.* (1993) was based on a *P. hispanus/P. divergens* sp. n. mixture. This would not be surprising as it is not unusual to find mixed species populations. However, the morphological diagnostic characters (shape and position of the vaginal sclerotisations, location of sperm in uteri in females, spicule shape and width and relative position of SP1 in relation to cloacal opening and SP2, in Portuguese populations, differs from the Spanish type population. A more detailed examination of a larger number of specimens of the trichodorids from Jaén is required to establish their specific identity.

Nematodes of the genus *Paratrichodorus* are natural vectors of *Tobacco rattle virus* (TRV). This virus has a wide range of serologically distinguishable strains and has the widest host range known of all plant viruses, causing serious damage in various economically important crops (Taylor & Brown, 1997). A strain of TRV was

transmitted by individual specimens of *P. hispanus* recovered from the rhizosphere of potatoes (Ploeg & Brown, 1997; Taylor & Brown, 1997) in the same farm of the type population of *P. divergens* sp. n. Posteriorly TRV was found in 7% of soil samples containing trichodorids, including *P. hispanus* as well as the newly described species in arable soils from areas where the virus might occur, in the Centre and North of Portugal (Duarte *et al.*, 2001, 2002). So correct identification is essential.

More recently, a PCR-RFLP method exploring the variability of the 18S rDNA gene to robustly discriminate trichodorid species belonging to *Trichodorus* and *Paratrachodorus* was developed for a rapid and accurate identification to species level (Duarte *et al.*, 2004). This molecular technique will provide an additional diagnostic tool in the future especially to distinguish closely related species, namely *P. divergens* sp. n. and *P. hispanus*.

Acknowledgements

We are very grateful to Mr João Basto from Granjas Farm, Esporões, Braga, for kindly allowing frequent sampling in his fields, to Eng. Sá Menezes for guiding us to various farms in the North, to Dr M. Arias from CSIC, Madrid, Spain, for providing type material of *P. hispanus*, to Mrs Conceição Queirós for helping with some specimen preparations, to Eng M.H. Vilhena and Prof. J. Paiva for identifying the plant species, to Laboratório de Sedimentologia, Departamento de Ciências da Terra, Universidade do Minho, Braga, for the sedimentological classification of soils, and to the SEM Laboratory, Centro de Materiais da Universidade do Porto, for providing the SEM and making available its facilities. The research was financed by Centro de Biologia (CB) da Universidade do Minho and partly done in facilities provided by Instituto do Ambiente e Vida (IAV), Departamento de Zoologia, Universidade de Coimbra.

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